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Biotic and abiotic factors affecting the $\delta^{13}C$ of soil respired CO$_2$ in a Mediterranean oak woodland†

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The flux ($R_s$) and carbon isotopic composition ($\delta^{13}C_{Rs}$) of soil respired CO$_2$ was measured every 2 h over the course of three diel cycles in a Mediterranean oak woodland, together with measurements of the $\delta^{13}C$ composition of leaf, root and soil organic matter ($\delta^{13}C_{SOM}$) and metabolites. Simulations of $R_s$ and $\delta^{13}C_{Rs}$ were also made using a numerical model parameterised with the SOM data and assuming short-term production rates were driven mainly by temperature. Average values of $\delta^{13}C_{Rs}$ over the study period were within the range of root metabolite and average $\delta^{13}C_{SOM}$ values, but enriched in $^{13}C$ relative to the bulk $\delta^{13}C$ of leaf, litter, and roots and the upper soil organic layers. There was good agreement between model output and observed CO$_2$ fluxes and the underlying features of $\delta^{13}C_{Rs}$. Observed diel variations of 0.5 ‰ in $\delta^{13}C_{Rs}$ were predicted by the model in response to temperature-related shifts in production rates along a $\sim$ 3 ‰ gradient observed in the profile of $\delta^{13}C_{SOM}$. However, observed $\delta^{13}C_{Rs}$ varied by over 2 ‰, indicating that both dynamics in soil respiratory metabolism and physical processes can influence short-term variability of $\delta^{13}C_{Rs}$.

Keywords: diurnal cycles; carbon-13; carbohydrates; isotope ecology; soil respiration model; Quercus suber

1. Introduction

The stable carbon isotope composition of soil CO$_2$ fluxes ($\delta^{13}C_{Rs}$) can provide an opportunity to study chemical, physical and biological processes at the soil–plant–atmosphere interface and understand biosphere isotopic signals at larger scales [1–3]. Carbon isotope signals provide integrated information on the metabolic processes of plants and soil heterotrophs, thereby providing a promising tool for partitioning the contribution of autotrophic and heterotrophic respiration.

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to soil and ecosystem net fluxes and elucidating how these contributions might change with environmental drivers or developmental cues (for review see [4]).

Soil respired CO₂ is primarily composed of a plant associated autotrophic (roots and rhizosphere micro-organisms) and a heterotrophic component associated with the mineralisation of organic matter [5]. The importance of canopy processes on the autotrophic component of soil respiration has been observed through variations in δ¹³Cₐₕ of more than 5 ‰ associated with weather changes at the synoptic scale [6,7] and soil water content at the seasonal scale [8]. Dynamic changes in microbial populations utilising different respiratory substrates can also contribute to variations in δ¹³Cₐₕ [9–11].

Mediterranean ecosystems have large seasonality in temperature and water availability, and phenomena such as the ‘Birch effect’, which involves rapid decomposition and microbial population changes following sporadic rain events under dry conditions [12], may result in significant short-term variability in δ¹³Cₐₕ. Variation of more than 4 ‰ was observed in the δ¹³C of night-time ecosystem respiration in a Mediterranean oak ‘montado’ woodland [13], but it is unknown to what extent this degree of variability is driven by plant [14,15] or soil respiratory processes. Diel (i.e. within the 24 h period) and day-to-day variability in δ¹³Cₐₕ of up to 2 ‰ has been observed in pine [16], beech [17], and eucalyptus [18] forests, indicating the potential for soil processes to contribute to short-term variability in the δ¹³C of ecosystem CO₂ fluxes. Considering the important role that isotopic data could provide for investigating carbon cycle processes [19], an understanding of processes affecting the δ¹³C of fluxes at short time scales is crucial for further applications such as partitioning net fluxes [2].

In this study, we have examined the diel variations in the δ¹³C signatures of the CO₂ fluxes from a Mediterranean oak-dominated woodland soil during a three day study in April 2005. We manually collected air samples from automated open soil chambers that were then analysed with respect to the mole fraction and isotopic composition of CO₂. We also measured soil properties (carbon content, root density, temperature, and moisture content) and collected foliage and soil samples to determine the isotopic composition of the carbon pools that are the potential substrates for the fluxes. The data were then used together with a soil CO₂ production and flux model to explore the variability of the isotope composition of the net fluxes on a diurnal and daily timeframe.

2. Materials and methods

2.1. Site description

The study took place in Herdade da Mitra (38°32′N, 8°01′W, 221 m a.s.l.) 12 km southwest of Évora in southern Portugal. The climate is typically Mediterranean, with most precipitation falling between October and April and a hot and dry summer. The experimental plot was on a 5° slope on acid litholic non-Humic soil with a pH of 4–6 [20]. The soil profile, characterised 500 m from the site [20], consisted of 89 % sand, 5 % silt, and 6 % clay in the upper 1 m, with a water retention capacity of 5 % (pF₂.₅ = 8 and pF₄.₂ = 3). The experimental plot encompassed an area of 0.264 ha (46 × 60 m) exclusively covered with Quercus suber L. trees planted in 1988, with an understorey composed of Cistus salvifolius L. and C. crispus L. and herbaceous plants (mostly winter-spring C₃ annuals).

At the end of the field campaign, a pit transect (3.6 m long × 1.4 m wide × 1.5 m deep) was dug nearby the experimental plot to characterise and quantify root biomass from the different vegetation types (grass, shrubs, and trees). From this transect, three soil profiles were collected vertically with samples every 0.1 m down to 1 m depth. The majority of root biomass was observed at 0.2, 0.4, and 0.9 m depth, with 19, 13, and 17 %, respectively, of the total root biomass [21].
2.2. Meteorological and flux measurements

Weather conditions were continuously recorded at a meteorological station set up at the field site. Precipitation, air humidity and temperature above the canopy were measured every 5 min, averaged and logged every 30 min to a data logger. Values of atmospheric vapour pressure deficit (VPD) were calculated from air temperature and humidity data. Soil water status beneath each soil chamber was monitored daily throughout the field campaign at depths of 10, 20, 30, 40, 60, and 100 cm using a PR2 Profile Probe attached to a HH2 moisture meter (Delta-T Devices Ltd., Cambridge, UK). Soil temperature was recorded at depths of 0, 2.5, 5, 10, 20, 30, and 100 cm and the average and SD of measured temperature stored every 15 min.

2.3. Soil surface CO\textsubscript{2} efflux

An automatic open chamber soil respiration system was deployed in the experimental plot, described in detail in Wingate et al. [3]. The soil chambers operated as an open gas exchange system, i.e., the net CO\textsubscript{2} flux was calculated from the difference in the CO\textsubscript{2} concentration between air flowing into and out of the chamber and the flow rate [3,22]. In brief, ambient air was pumped through the chamber reference and sample lines at a flow rate of 1 dm\textsuperscript{3} min\textsuperscript{-1}, passed through drying columns containing magnesium perchlorate within 1 m of the chamber to prevent condensation of water vapour in the lines and delivered to an infra-red gas analyser for the determination of CO\textsubscript{2} concentration (CIRAS-DC, PP Systems, Hitchin). Between measurements, the pneumatically-actuated chambers were raised above the soil surface, to minimise disturbances in the water and energy balance of the soil inside the chamber. Each chamber was sampled for 15 min.

2.4. $\delta^{13}C$ of soil surface CO\textsubscript{2} efflux

Reference and sample air was collected every 2 h for about 72 h from two soil chambers. A glass flask of 0.2 dm\textsuperscript{3} was placed in-line on each air stream immediately after the soil chamber ($\leq$1 m) and a drying column containing magnesium perchlorate. Tubing was inserted inside a glass side-arm and secured to Cajon fittings that allowed complete flushing of the flasks (see Hemming et al. [23] for full description). Flasks were placed in-line before the soil chamber lid closed and flushed with the air stream until about 20 s prior to the chamber opening, at which point the inner tubes were retracted from the flasks and the vacuum stopcocks closed. The $\delta^{13}C$ of CO\textsubscript{2} in air was then analysed on a Europa 20-20 isotope ratio mass spectrometer (IRMS) (Crewe, UK) at the Weizmann Institute of Science in continuous flow configuration [23]. A working standard was analysed for every five samples, with the precision (SD) of the standards within a run being $\pm 0.1\%$.e.

2.5. Calculations of $\delta^{13}C$ signatures of soil CO\textsubscript{2} exchange

During the 15 min chamber closure period, steady-state gas exchange was verified visually in the field using a laptop connected to a data-logger before the glass flasks were taken and the chambers re-opened. The carbon isotope signal of the net CO\textsubscript{2} fluxes during chamber closure ($\delta^{13}C_{Rs}$) was calculated using a simple isotopic mass balance:

$$
\delta^{13}C_{Rs} = \frac{\delta^{13}C_o C_o - \delta^{13}C_e C_e}{C_o - C_e},
$$

(1)
where \( C_o, C_e, \) and \( \delta^{13}C_o, \delta^{13}C_e \) are the mole fractions and isotopic compositions of CO\(_2\) in the air leaving and entering the chamber, respectively.

2.6. Photosynthesis, stomatal conductance and photosynthetic discrimination

On six representative \( Q. \) suber trees (three trees from two plots), measurements of net photosynthesis (\( A \)) and stomatal conductance (\( g_s \)) were made at three points during the hours of 10:00 and 16:00 during the field campaign. Gas exchange measurements were performed with an open flow gas exchange system (Li-6400, Licor Inc., Lincoln, NB, USA). Measurements were made under ambient radiation and atmospheric conditions, and readings taken when steady-state conditions were reached in the leaf cuvette, being when the 30 s co-efficient of variation for cuvette CO\(_2\) and H\(_2\)O concentrations was less than 1%.

From the gas exchange data, values of predicted photosynthetic \(^{13}\)C discrimination (\( ^{13}\Delta_{\text{pred}} \)) were calculated, using an estimate of mesophyll conductance (\( g_m \)) and values of \( A \) to estimate the CO\(_2\) concentration in the chloroplast [24,25]:

\[
^{13}\Delta_{\text{pred}} = a + (b - a) \frac{C_i}{C_a} - (b - a_m) \frac{A}{g_m} - f \frac{\Gamma^*}{C_a},
\]

where \( a, b, a_m, \) and \( f \) are the fractionation factors associated with diffusion (4.4 ‰), carboxylation (29 ‰), mesophyll transfer (1.8 ‰) and photorespiration (8 ‰), respectively, \( C_i \) and \( C_a \) are the CO\(_2\) concentrations in the leaf intercellular space and in air, respectively, and \( \Gamma^* \) is the CO\(_2\) compensation point in the absence of photorespiration. Mesophyll conductance was not measured directly, but values of between 0.07 and 3 mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\) have been measured for Quercus species [26].

2.7. Bulk soil organic matter \( \delta^{13}C \) composition

Soil samples were collected at four depths (0–5, 5–10, 10–15, 15–20 cm depth) in the vicinity (within 30 cm) of the soil chambers. The samples were dried and ground and treated with 0.5 M HCl for 24 h to remove any carbonates [27]. Following carbonate removal samples were weighed into tin cups for carbon content and bulk \( \delta^{13}C \) analysis using an online combustion elemental analyser (EA1109 CHN-O; Carlo Erba Instruments, Milan, Italy) connected to an IRMS (Optima; Micromass, Manchester, UK). Four samples of the acetanilide elemental international standard (NIST traceable, Elemental Microanalysis Ltd., #B20CC) were included in each run, and a correction applied to account for the influence of a blank cup. Two replicates of each sample were analysed and two samples of a cellulose laboratory working standard were measured for every 12 samples. The precision of each replicate analysis of the working standard was \( \pm 0.1 \) ‰.

2.8. Foliage and root metabolite \( \delta^{13}C \) composition

Leaf samples were collected from around the circumference of the crown of the \( Q. \) suber trees and \( Cistus \) shrubs in the late afternoon on each day. Five to ten samples were collected at each time from oaks in both plots 1 and 2 and \( Cistus \) in plot 1 and immediately frozen in liquid nitrogen. Samples of surface litter and root litter from sieved soil samples were collected from each plot at the conclusion of the campaign. Soluble sugars were extracted and purified from these samples following a procedure adopted from Tcherkez et al. [28]. In brief, 50–100 mg of powdered leaf, surface litter or root material was suspended with 1 ml of cold distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany), maintained in ice for 60 min and vortexed every 10 min. After centrifugation at 14,000 g for 15 min at 5 °C, the supernatant was
carefully retrieved, and the pellet was maintained at −20 °C for starch extraction. The supernatant, containing the water soluble fraction, was then heated at 100 °C for 5 min and kept in ice for 30 min to precipitate the heat-denatured proteins. After centrifugation at 14,000 g for 15 min at 5 °C to remove the protein pellet, the protein-free water-soluble fraction was maintained at −20 °C for further extraction of individual soluble sugars by high-pressure liquid chromatography (HPLC) [29]. The water soluble fraction was filtered (filter HV 0.45 μm type; Nihon Millipore Kogyo, Osaka, Japan) before injection to HPLC. For each sample, 100 μl of filtered extract was injected into a Sugar-Pak1 column (6.5 mm diameter and 300 mm length; Waters, Milford, MA, USA) and individual sugar peaks (sucrose, glucose, and fructose), as well as a large peak (containing mainly organic acids and amino acids) eluted prior to the sucrose peak, were collected and frozen in liquid nitrogen. In the Cistus samples, fructose was co-eluted with myoinositol and glucose was co-eluted with an unknown metabolite, and these additional metabolites were collected together with the sugars. These extra-peaks were not observed for oak leaf samples. After lyophilisation, purified sugars were suspended in distilled water, transferred into tin capsules (Courtage Analyses Services, Mont Saint-Aignan, France) and dried at 50 °C for isotope analysis.

Starch was extracted following Duranceau et al. [29]. Briefly, the pellet containing the water insoluble fraction was first washed three times with 1 ml 95 vol % ethanol at 0 °C, in order to remove the pigments. The pellet was then suspended twice with 1 ml 6 N HCl, transferred to a 10 ml tube, and kept 1 h at 5 °C to solubilise the starch. After 20 min centrifugation at 14,000 g and 5 °C, the supernatant comprising the solubilised starch was retrieved and poured into a 50 ml tube and another 1 ml 6 N HCl was added to the pellet to repeat the preceding step, and recover the remaining starch. Methanol (four times the total supernatant volume) was added to the tube containing the supernatant extracted with the preceding steps. This mix was kept over night at 5 °C to precipitate starch. Finally, after centrifugation at 14,000 g for 15 min and 5 °C, the supernatant was removed and the precipitated starch was freeze-dried for isotopic analysis.

Carbon isotope analysis of the carbohydrates and bulk organic matter was performed on an IRMS (VG Optima; Micromass, Villeurbanne, France) connected to an elemental analyser. A glutamic acid laboratory standard (traceable to an IAEA glutamic standard) was measured every 12 samples, with precision of ±0.08 ‰ (SD of 5 standards in a run). All δ13C values are relative to the V-PDB standard.

2.9. Model description

We used a one-dimensional numerical model (21 layers of increasing thickness, 1 m total depth) that simulates soil CO2 production and diffusion and the resulting concentrations of 12CO2 (Cs) and 13CO2 (in μmol m−3) in the soil [3,30]:

\[
\frac{\partial((\theta_a + B \theta_w) C_s)}{\partial t} = \frac{\partial}{\partial z} \left( D_s \frac{\partial C_s}{\partial z} \right) + S_c, \tag{3}
\]

\[
\frac{\partial((\theta_a + B \theta_w) \Re_s C_s)}{\partial t} = \frac{\partial}{\partial z} \left( D_{13} \frac{\partial(\Re_s C_s)}{\partial z} \right) + \Re_c S_c, \tag{4}
\]

where \(\theta_a\) and \(\theta_w\) (m3 m−3 soil) are the soil air and water content, \(B\) (m3 air m−3 water) is the temperature-dependent Bunsen solubility coefficient, \(S_c\) (μmol m−3 s−1) is the CO2 production rate dependent on time \(t\) and depth \(z\), and \(\Re_s\) and \(\Re_c\) are the 13C/12C ratios of CO2 in soil air and respired CO2, respectively. The effective diffusivity of CO2 in soil air, \(D_s\) (m2 s−1) was calculated as [31]

\[
D_s = D_{25} \theta_a^2 \left( \frac{\theta_a}{\theta_{sat}} \right)^{3/\beta} \left( \frac{T_s}{T_{25}} \right)^n, \tag{5}
\]
where \( D_{25} \) is the molecular diffusivity of CO\(_2\) at 25 °C (1.41 \(-5 \) m\(^2\) s\(^{-1}\)), \( T_s \) (K) is the soil temperature, \( \theta_{\text{sat}} \) is the saturated water content, \( n \) is 1.5 [32], and \( \beta \) is the slope of the water retention function, estimated here as 4.9 [21]. In Equation (3), \( D_s^{13} = D_s \alpha_d \) is the diffusivity of \(^{13}\)CO\(_2\) in soil air, where \( \alpha_d = 1 + \varepsilon_d \), using the kinetic isotope fractionation \( \varepsilon_d \) of −4.4 ‰ [33].

The net fluxes of \(^{12}\)CO\(_2\) and \(^{13}\)CO\(_2\) were then computed from their simulated gradients at the soil surface. This yields the \( \delta^{13} \)C composition of the flux without a chamber, which differs slightly (<0.1 ‰) from that observed in a chamber.

We used profile data on soil temperature and water content concurrently collected at the field site. Soil CO\(_2\) production was parameterised using an exponential profile with e-folding depth of 10 cm and decreasing rates in the dry layers between 5 cm depth and the soil surface to account for the vertical distribution of soil water content [3] and root biomass [21]. We assumed that the short-term variation (i.e. for time scales less then the experiment duration) in production is driven predominantly by temperature changes. The CO\(_2\) production rate \( p_s(t, z) \) was then modified based on profile data of soil temperature as [34]

\[
p_s(t, z) = p_n(z) p_0 e^{E_o((1/56)-(1/T_{\text{sat}}(t,z)+46))},
\]

where \( p_0 \) is the CO\(_2\) production rate at 10 °C, and \( E_o \) is the activation energy of soil CO\(_2\) production. For the \( \delta^{13} \)C of CO\(_2\) produced in the soil, we fitted an exponential profile to the observed \( \delta^{13} \)C of soil organic matter (SOM) (Figure 3a). The soil \(^{12}\)CO\(_2\) and \(^{13}\)CO\(_2\) profiles were initialised with steady-state solutions, and transient calculations were then performed on a time step of 60 s.

3. Results

3.1. Meteorological conditions

The sampling campaign was conducted three to four days after a 9 mm rain event during warm, dry and clear conditions (Figures 1a and b). Over the sampling period, the average day-time (between 10:00 and 18:00) VPD ranged between 0.95 and 1.34 kPa. Beneath one of the soil chambers (chamber 1) soil water content was 4–6 % over the top 40 cm, increasing at lower depths to 20 % at 1 m, while beneath the other chamber (chamber 2) soil water content increased from 8 % at 10 cm depth to 18 % at 40 cm, and decreased slightly (to 15 %) at 1 m [3]. Soil temperatures in the top 10 cm showed strong diel oscillations of an amplitude of 15 °C or more that decreased to 1–2 °C at 40 cm and less than a degree at 1 m (Figure 1b).

3.2. Soil surface CO\(_2\) mole fraction and \( \delta^{13} \)C composition

The concentration and \( \delta^{13} \)C composition of CO\(_2\) measured in the reference line of the chambers exhibited diel patterns consistent with varying contributions of turbulent mixing and respiratory CO\(_2\) on the composition of soil surface air (Figures 2a and b). Calm conditions persisted during the first and third nights of the sampling campaign, promoting a conspicuous build-up of respired CO\(_2\) where concentrations reached values of \(~480\) ppm. However, on the second night, turbulent conditions kept the air well mixed at atmospheric background concentrations. The same pattern was observed in the carbon isotopic composition of ambient air (\( \delta^{13} \)C\(_a\)) with values of −8.5 ‰ during the day falling to −12 ‰ during the calm nights as the contribution of depleted respiratory CO\(_2\) built up.
Figure 1. Relative humidity and precipitation measured prior to and during the isotope field campaign (a), and air and soil temperature at various depths in the soil profile (b).

Figure 2. Time-series observations of (a,b) flask CO$_2$ mole fraction (circles) and the carbon isotope composition of flask CO$_2$ ($\delta^{13}$C$_a$, triangles) collected in the soil chamber reference air stream; the measured (circles) and model predicted (lines) of (c,d) soil CO$_2$ flux ($R_s$) and (e,f) carbon isotope composition of the soil CO$_2$ flux ($\delta^{13}$C$_{RS}$) measured in chamber 1 (filled circles; a,c,e) and chamber 2 (open circles; b,d,f) during the field campaign. Vertical shading indicates night-time periods.
3.3. **Soil CO₂ efflux rates and δ¹³C composition**

A clear diel pattern was observed in the soil CO₂ flux ($R_s$) for both chambers that followed changes in soil temperature closely (Figures 2c and d). Maximal respiration rates were slightly higher in chamber 2 while minimum rates were the same.

The δ¹³C of the soil CO₂ efflux (δ¹³C$_{Rs}$) did not differ significantly between the two plots (Figures 2e and f, Table 1). The average δ¹³C$_{Rs}$ for this site was $-25.9\%\text{e}$ (SD = 0.5, $n$ = 71, both plots combined). However, δ¹³C$_{Rs}$ varied by over 2% during the sampling period, from between $-27.1\%\text{e}$ and $-25.8\%\text{e}$ (Figures 2e and f). In contrast to respiration rates, the δ¹³C$_{Rs}$ variation did not show an obvious diel pattern. Thus we could find no clear relationship between the rate of respiration and its isotopic composition. These δ¹³C$_{Rs}$ values were similar to those measured in a previous campaign during September 2004 at the site using two different chamber methods ($-26.1\pm0.9\%\text{e}$ SD, $n$ = 17, for a closed chamber, and $-26.1\pm0.6\%\text{e}$ SD, $n$ = 20, for the open chamber described above).

3.4. **Soil profile carbon content and isotopic composition**

The SOM ¹³C composition (δ¹³C$_{SOM}$) became progressively enriched in both plots by 2.5–3% over the top 20 cm, (from $-27.3$ to $-24.6\%\text{e}$ for the average of the two plots, Figure 3a), but overall differences between plots were not large, with respective carbon content weighted δ¹³C$_{SOM}$ values of $-26.7$ and $-25.9\%\text{e}$ (indicated by arrows in Figure 3a). Soil carbon content decreased with depth in both plots and was higher in plot 1, but the plot difference decreased with depth (Figure 3b). Soil carbon content did not vary much over the lower layers, and average carbon content between 5 and 20 cm was 0.9 and 0.4 % in plots 1 and 2, respectively.

3.5. **Numerical modelling of soil fluxes and δ¹³C**

Using the numerical model described above, we investigated the response of the soil CO₂ flux and its δ¹³C composition to changes in environmental conditions. We assumed that short-term variations in the soil CO₂ production rate are mainly driven by changes in soil temperature. As a consequence, the spatial and temporal patterns of CO₂ production (Figure 4b) are similar to those of the soil temperature (Figure 4a): a decreasing diurnal amplitude towards the bottom of the profile, and an increasing lag in the timing of production maxima and minima, with a lag of several hours between the soil surface and 20 cm depth. In contrast, soil CO₂ concentration (Figure 4c) and the δ¹³C of soil CO₂ (Figure 4d) both had variations of similar amplitudes and timing at all depths.

### Table 1. Mean carbon isotope composition of organic matter and fluxes in the oak woodland measured over three days.

<table>
<thead>
<tr>
<th></th>
<th>δ¹³C$_{Suc}$ (%)</th>
<th>δ¹³C$_{Gluc}$ (%)</th>
<th>δ¹³C$_{Fru}$ (%)</th>
<th>δ¹³C$_{Starch}$ (%)</th>
<th>δ¹³C$_{Leaf}$ (%)</th>
<th>¹³Δ$_{pred}$ (%)</th>
<th>δ¹³C$_{Rs}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plot 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$Q. suber$</td>
<td>$-26.7 (0.5)$</td>
<td>$-27.8 (-)$</td>
<td>$-24.3 (0.4)$</td>
<td>$-26.3 (1.2)$</td>
<td>$-29.1 (0.4)$</td>
<td>$20.5 (1.3)$</td>
<td>$-25.8 (0.9)$</td>
</tr>
<tr>
<td>$Cistus$</td>
<td>$-27.7 (1.0)$</td>
<td>$-30.1 (0.5)$</td>
<td>$-32.5 (0.5)$</td>
<td>$-27.2 (0.5)$</td>
<td>$-29.7 (0.7)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plot 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Q. suber$</td>
<td>$-26.5 (0.9)$</td>
<td>$-27 (0.6)$</td>
<td>$-24.4 (0.2)$</td>
<td>$-25.9 (0.4)$</td>
<td>$-28.8 (0.7)$</td>
<td>$21.2 (1.0)$</td>
<td>$-25.9 (0.5)$</td>
</tr>
</tbody>
</table>

Notes: The δ¹³C composition of carbohydrates (Suc: sucrose, Gluc: glucose, Fru: fructose) and bulk leaf organic matter was measured in *Quercus suber* L. and *Cistus* ssp. and the isotopic discrimination predicted from gas exchange measurements of $A$ and $g_s$ (¹³Δ$_{pred}$) was measured in *Q. suber* trees. The δ¹³C composition of soil respired CO₂ (δ¹³C$_{Rs}$) was measured in two plots. Values are mean (SD) of all measurements in the three days.
Variations in modelled CO2 fluxes at the soil surface were also related to soil temperature, reflecting changes in regional meteorological conditions at the synoptic time scale (Figure 3c). After an initial decline in soil temperatures and CO2 fluxes due to the rain events of 2nd and 3rd April, increasing soil temperatures resulted in larger CO2 fluxes with a maximum around the time of the isotope sampling period. Modelled fluxes agreed well with the observed fluxes during the sampling period (Figures 2c and d). Similarly, the modelled δ13C of the soil CO2 fluxes showed diurnal variations, but only varied by up to 0.5 ‰, much less than the range of variation observed in δ13C_Rs (Figures 2e and f and Figure 3c).

Under transient conditions, the CO2 flux at the soil surface and its δ13C signature can differ from the depth-integrated soil CO2 source and its δ13C (not shown). Flux and depth-integrated sources are only equal for the steady-state conditions at the beginning of the simulation. At all subsequent time steps, changes in soil CO2 production take time to propagate through the soil profile, thus soil fluxes appear slightly dampened (by up to 0.2 μmol m⁻² s⁻¹, and 0.1 ‰ for δ13C) compared to the integrated sources.

As a sensitivity test, shifting soil production to shallower or deeper soil layers (shown in Figure 3b) changed the average values of δ13C_Rs as well as their diel amplitudes (Figure 3c). For example, shallower production resulted in more depleted day-time δ13C_Rs values without affecting the enriched night-time flux signatures, creating stronger diel variations.

3.6. Gas exchange measurements and photosynthetic discrimination

Rates of assimilation and stomatal conductance on Q. suber trees were also found to be similar between plots. Average A was 8.7 (±2.1 SD) μmol m⁻² s⁻¹ and average gs was 0.18 (±0.06 SD) mol m⁻² s⁻¹ across all measurements (all trees and both plots). From these measurements, we predicted that the average photosynthetic discrimination (13 Δ_pred, Equation (2)) was 18.2 ‰ (±0.8) (Table 1, assuming a g_m of 0.2 mol m⁻² s⁻¹ bar⁻¹). There were good relationships between
$g_s$ and VPD (an exponential decline, with regression $r^2$ of 0.95) and between $A$ and $g_s$ (asymptotic exponential, $r^2 = 0.87$) seen in the gas exchange data. Thus, assimilation rates, stomatal conductance and $^{13}\Delta_{pred}$ could be approximated for the sampling period, and the $\delta^{13}C$ composition of photoassimilates ($\delta^{13}C_{phs}$) was estimated from the measurements of $\delta^{13}C_a$. The average value of $\delta^{13}C_{phs}$ for the six day period (including three days prior to the isotope sampling) ranged
between $-26.3\%$ ($\pm 0.1$) and $-28.6\%$ ($\pm 0.1$), for a range of $g_m$ values between 0.15 and 0.25 mol m$^{-2}$ s$^{-1}$, respectively.

3.7. Plant metabolite and organic matter $\delta^{13}C$

Both the bulk leaf organic matter and leaf metabolites from the Cistus leaves were $^{13}C$ depleted compared to the corresponding components from the Q. suber trees, but the relationship between metabolite and bulk leaf differed between the species (Table 1). There was no statistical difference between the Q. suber bulk leaf $\delta^{13}C$ values between the two plots, but there was a small ($0.6\%$) difference between the $\delta^{13}C$ of the bulk leaf of the Cistus plants and oak trees ($p < 0.05$). All metabolites were $^{13}C$-enriched relative to the bulk material for those extracted from the Q. suber leaves, while the fructose and glucose from the Cistus leaves were the most depleted components measured in the system (Table 1). The depletion of these sugars was mainly due to the co-elution of fructose with a $^{13}C$-depleted metabolite, i.e. myo-inositol (Tcherkez and Ghashghaie, unpublished data), and the co-elution of glucose with an unknown, and probably $^{13}C$-depleted, metabolite. These co-eluting peaks were not observed for Q. suber leaf samples. The similarity in bulk leaf $^{13}C$ composition between the Cistus and Q. suber plants indicates that the amount of metabolites present (at most a few percent of total leaf mass, data not shown) were not enough to influence the composition of the bulk leaves and litter in the system (see below).

The day-to-day variation in the $\delta^{13}C$ composition of sucrose ($\delta^{13}C_{Suc}$) over the course of the sampling was between 0.4 and 1.6\%, depending on species and plot (Table 2). Fructose from the Q. suber leaves was the most $^{13}C$ enriched component and was ca. 2–3\% enriched relative to the sucrose and glucose. In addition, the additional soluble compounds (collected just before the sugar peaks and containing mainly organic and amino acids) extracted from the Cistus and Q. suber leaves had $\delta^{13}C$ compositions of $-28.9 \pm 1.0\%$ ($n = 6$) and $-28.6 \pm 0.4\%$ ($n = 11$), respectively (data not shown). Overall, the $\delta^{13}C$ composition of the various organic components in the system covered a range of over 8\%.

Figure 5 summarises the $\delta^{13}C$ values of the various pools and fluxes measured in this study (showing values for the Q. suber leaves only, and with values for both plots pooled for clarity). The bulk surface litter $\delta^{13}C$ values were very similar to those of the oak leaves, while the $\delta^{13}C$ of the deeper root litter was ca. 1.5\% enriched compared to the surface litter (Figure 5). The soluble sugars from the litter and roots were also isotopically enriched relative to the bulk material, but covered a similar range to that of the SOM. There was a general isotopic enrichment in the labile compounds in the system going from the canopy (the estimated assimilate values and leaf carbohydrates) to the roots and litter (Figure 5). At the same time, the $\delta^{13}C_{Rs}$ was similar to the SOM and soluble sugar compounds. For comparison we also show in Figure 5 the annual average $\delta^{13}C$ values from organic material and the CO$_2$ of ecosystem respiration measured at the near-by

<table>
<thead>
<tr>
<th>Day</th>
<th>Q. suber</th>
<th>Cistus</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 April</td>
<td>$-26.5 (0.2)$</td>
<td>$-26.8 (0.7)$</td>
</tr>
<tr>
<td>8 April</td>
<td>$-26.9 (0.5)$</td>
<td>$-28.3 (0.7)$</td>
</tr>
<tr>
<td>9 April</td>
<td>$-26.7 (0.7)$</td>
<td>$-28.5 (0.3)$</td>
</tr>
</tbody>
</table>

Notes: Values are mean (SD) of all measurements on each day.
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Figure 5. A comparison of the δ¹³C composition of the various pools and fluxes measured in the oak woodland. Leaf values are for Q. suber trees only. Data from both this study and measurements in September 2004 are shown for the SOM and soil respired CO₂. The assimilate value is the estimated six day mean value including the three days prior to the sampling period with the error bars representing the range of estimates based on a range of mesophyll conductance values between 0.15 and 0.25 mol m⁻² s⁻¹ bar⁻¹. Leaf, root and litter bulk and metabolite data are the mean (±SD) values for all samples collected over the three day period. Soil respired CO₂ values are the mean (±SD) for all measurements in each campaign and SOM values are the carbon content weighted means across the profile (±SD of the two plots). Also shown (white symbols; letter W in the legend) are the mean (±SD) values of components measured through the course of one (organic matter, five to eight measurement dates), and two (ecosystem respiration, measurement 25 dates) years at a nearby flux site [13].

CarboEurope flux site, Mitra (see Table 1 and Figure 1 in Werner et al. [13]). Bulk leaf δ¹³C values (Q. ilex in the adjacent site) were similar between the two oak species, and again there was a ¹³C enrichment between canopy and the SOM values. Ecosystem respiration δ¹³C values covered a range of more than 7 ‰ and averaged −28.4 ± 2 ‰ [13].

4. Discussion

4.1. δ¹³C Rs is similar to soil carbon pool δ¹³C but displays short-term variability

Through monitoring soil CO₂ efflux at high temporal resolution over three contiguous diel cycles, we observed that the values of the δ¹³C composition of soil CO₂ efflux in this Mediterranean woodland were consistent with δ¹³C values of soil carbon pools (SOM and root labile carbon compounds, Figure 5), but also varied by more than 2 ‰, often within the space of a few hours (Figure 2). Observations of the soil efflux isotopic composition at sub-daily resolution are still quite scarce, but variations of a similar magnitude have been observed from other soils over the diel scale in a Pinus sylvestris L. forest [16] and a Fagus sylvatica L forest [17], and day-to-day scale (0.5–2 ‰) in a Eucalyptus forest [18]. In contrast, little diurnal variation was observed in a boreal conifer system during long summer days [35]. However, while the rate of soil CO₂ efflux
appeared to be driven, to a large extent, by the diel soil temperature oscillations, there was no clear diel pattern in the δ¹³C signal evident in our system in contrast to the *P. sylvestris* or *F. sylvatica* forests [16,17].

This short-term variability in the δ¹³C<sub>Rs</sub> signal (a total range of 2.6‰) is of interest when making inferences about below-ground carbon cycle processes and using such data for flux partitioning at the ecosystem level [2,19]. We found that both biological and physical factors may underlie the observed variability. Soil surface CO₂ efflux has complex origins due to a range of respiratory sources, including plant roots and microbial populations, and variable depths of production with differences in the associated sources and transport times to the surface. The similarity between the range of δ¹³C<sub>Rs</sub> values to both root sugar and SOM δ¹³C values (Table 1, Figure 3a) makes it impossible to separate the contributions of the heterotrophic and autotrophic components of the soil CO₂ efflux. Evidence from the net δ¹⁸O composition of the soil CO₂ efflux measured during the same campaign [3] indicate appreciable microbial activity and the photosynthetic rates indicate a potential supply of photoassimilates available for transport and other metabolic processes. Therefore, the dynamics of both sources are probably contributing to the short-term variability we observed in the net CO₂ flux and its δ¹³C composition.

Our values of soil respired δ¹³C tended to be enriched relative to canopy leaves and litter, and there was a progressive δ¹³C enrichment in organic material between the canopy and soil (Figure 5), which is a general feature of carbon pools at the ecosystem level [19]. However, whilst δ¹³C<sub>Rs</sub> was enriched relative to the δ¹³C of bulk leaf and the upper layer of SOM, it was also within the range of SOM δ¹³C values observed across the profile, that displayed a typical ~3‰ enrichment with depth (Figure 3a). Thus, we propose that δ¹³C measurements of bulk SOM composition integrated across the appropriate depth profile can provide a good approximation of the δ¹³C of soil CO₂ efflux in these Mediterranean ecosystems.

Root respired CO₂ is closely linked with above ground processes, and there is evidence that the photosynthetic activity of canopies in the previous zero to six days can have a strong influence on Rs and the associated δ¹³C composition [6,36,37]. This recently assimilated C can represent a large portion of the fast turnover C pools of labile substrates contributing to soil respiration [38,39]. Through the effects on stomatal conductance and variations in the extent of isotopic discrimination during photosynthesis, short-term changes in temperature and humidity can result in leaf-level shifts in the isotope composition of sugars and phloem organic matter transported to the roots and soil. Day-to-day variations in δC<sub>Suc</sub> were up to 1.7‰ over our sampling period (Table 2), similar to variations in the δ¹³C of twig phloem for different species over diurnal and day-to-day time frames [16,18]. However, canopy level variations in discrimination probably did not contribute substantially to the observed variations in δ¹³C<sub>Rs</sub> as they tend to be dampened during transport through the tree [16,35], hence greater than 2‰ shifts in δ¹³C<sub>Phs</sub> would be required to substantially contribute to the variation in δ¹³C<sub>Rs</sub>.

The δ¹³C<sub>Phs</sub> values derived from the gas exchange data were similar to the δC<sub>Suc</sub> values only if considerations of mesophyll conductance (g<sub>m</sub>) were included [24,25,40], otherwise estimated δ¹³C<sub>Phs</sub> values were 2–3‰ more depleted. However, sensitivity to g<sub>m</sub> was high, and a change in g<sub>m</sub> of 0.1 mol m<sup>−2</sup> s<sup>−1</sup> bar<sup>−1</sup> resulted in an increase in $\Delta_{\text{pred}}^{$ of over 2‰ (Figure 5). Thus, any short-term (minutes to hours) changes in g<sub>m</sub> [26] may also contribute to the δ¹³C variability of the carbon entering the system [40] in addition to the well-known effects of stomatal conductance and assimilation rate.

### 4.2. Soil profile δ¹³C and temperature influence on δ¹³C<sub>Rs</sub> variability

The δ¹³C enrichment in SOM with depth, in our case nearly 3‰ over the top 40 cm, is a common but not entirely understood feature of forest ecosystems [4,8,10,41]. We found that this pattern
can contribute to the variability in $\delta^{13}C_{Rs}$. Incorporating the $\delta^{13}C_{SOM}$ depth changes, together with the temperature driven diurnal variations in soil CO$_2$ production at the different depths in the soil CO$_2$ model, we showed that shifting contributions from different depths can create diel oscillations of $0.5\,\%e$ or more in $\delta^{13}C_{Rs}$.

The simulated soil CO$_2$ production rate showed strong spatial and temporal patterns (Figure 4b), with decreasing amplitude and an increasing delay of production maxima and minima towards the bottom of the profile associated with the temperature changes. In contrast, the temporal variations in soil CO$_2$ concentration and $\delta^{13}C$ (Figures 4c and d) were nearly uniform and synchronous across the profile. This is because the soil gradients in CO$_2$ and $^{13}$CO$_2$ adjust very quickly across depth in this porous soil, so that the soil CO$_2$ and $\delta^{13}C$ profiles reflect the depth-integrated CO$_2$ source rather than the local fluctuations in production rates. The fluctuations in $\delta^{13}C$ of soil CO$_2$ were also associated with the changes in total soil CO$_2$ production as there was no variation in the $\delta^{13}C$ of CO$_2$ produced in a given layer over time. The largest temperature and production variations occurred in the upper layers with relatively depleted $\delta^{13}C$ values. Hence, when the soil CO$_2$ flux was high due to increased production in the upper layers, the flux became more depleted in $^{13}$C. Conversely, when production was low there was a greater influence of the more isotopically enriched deeper soil layers. As fluctuations in soil temperature were strongest at the soil surface, the diel variations in $\delta^{13}C_{Rs}$ further increased when production was shifted to shallower layers (Figure 3c). Diel oscillations with similar amplitudes of $0.5\,\%e$ to $1.5\,\%e$ (but enriched during high fluxes) are also evident in recently published high-resolution (30 min) time series data of $\delta^{13}C_{Rs}$ in the study of [17].

Good agreement between the model output and observed CO$_2$ flux rates (Figures 3a and b) was obtained by assuming that short-term variation in production rates were driven by temperature. The predicted $\delta^{13}C_{Rs}$ values also seemed to capture the underlying features of the flux $\delta^{13}C$ signature, indicating that diurnal variations in $\delta^{13}C_{Rs}$ can result from abiotic processes when there is a change in $\delta^{13}C$ of source CO$_2$ with depth, but independent of any temporal changes in the $^{13}$C composition of the production source (at a given depth). A recent numerical analysis based on a constant production rate and $\delta^{13}C$ over the soil profile also found that variation in the $\delta^{13}C$ of soil CO$_2$ efflux can arise from diel oscillations in the production rate and with variations in soil diffusivity [42]. Possible changes in the contribution of differing metabolic sources driven by response to environmental variables was proposed to account for the short-term variation in $\delta^{13}C_{Rs}$ observed in the studies of Kodama et al. [16] and Marron et al. [17]. Here we demonstrate that abiotic features must also underlie the diel variations in $\delta^{13}C_{Rs}$ associated with spatial variations in the $\delta^{13}C$ of CO$_2$ produced in the soil profile. Abiotic influences have also been shown to contribute to the observed links between $R_s$ and canopy processes due to diffusion processes having similar time scales as the canopy-soil time lags [43]. Clearly, quantifying the role of physical processes is crucial for interpreting the short-term variation in soil CO$_2$ fluxes and its isotopic composition, an issue that is likely to become more pertinent as emerging laser technology provides high resolution data sets [17].

4.3. Other sources of variability

The variability in the observed $\delta^{13}C_{Rs}$ was larger than could be attributed to the depth associated variation in SOM or potential photosynthetic inputs, indicating other sources of variation. Isotope fractionation effects during respiration can introduce further variability, and while Klumpp et al. [44] found little fractionation associated with root respiration, others [45,46] reported a $^{13}$C depletion of ca. $1\,\%e$ relative to sugars. In many cases, microbial respiration had a similar isotopic composition to bulk SOM [4,9–11], but both $^{13}$C enrichment and depletion of many $\%e$ relative to SOM has also been observed due to changes in labile substrate availability [47,48], temperature [9] and depth [10]. However, much of this apparent isotope fractionation in relation to bulk SOM
values was attributed to changes in population structure and the use of different substrates [9–11]. Microbial biosynthesis tends to result in $^{13}$C enriched biomass and $^{13}$C depleted CO$_2$ being released from the catabolic reactions [11,49]. Microbial biosynthesis may well have been a feature of this system, in response to the rain pulse a few days prior to the sampling period [12]. Furthermore, there are widespread differences in isotopic composition between different compounds, such as those observed across the metabolites in our samples (Table 2), as a consequence of non-statistical intra-molecular isotope distributions and enzymatic kinetic isotope effects during branch point metabolism [49–52]. The synthesis of $^{13}$C depleted compounds, such as lipids and lignin and the release of terpenes, or $^{13}$C enriched compounds, such as starch and the release of $^{13}$C-enriched leaf respired CO$_2$, results in an opposite effect on the remaining precursors that may then form the basis of subsequent respiratory substrates or, in the case of plant leaves, be exported to the stem and roots. These metabolism-related isotopic enrichment processes contribute to the widespread progressive $^{13}$C enrichment seen in compounds and bulk organs between leaves and roots also seen in this system [19,45,50]; see Figure 5. The synthesis of particular compounds at any point in time, and the consequent impacts on carbon pools and respired CO$_2$, will depend on cellular requirements influenced by a multitude of factors including growth stage and stress responses [11,19,53]. It is, therefore, plausible that wide ranging metabolic activities from the different components of a complex system like the soil and variable substrate use by microbes can create short-term fluctuations in isotopic signature of the respired CO$_2$ around the mean values of root metabolites or SOM $\delta^{13}$C values.

5. Conclusions

This study investigated the isotopic composition of soil respired CO$_2$ during an intensive series of measurements over the course of three contiguous diel cycles in a Mediterranean oak woodland. The average value of the $\delta^{13}$C$_{Rs}$ was similar to the $\delta^{13}$C of root sugars and was also within the range of $\delta^{13}$C$_{SOM}$ values, indicating the potential of these components to provide a measure of $\delta^{13}$C$_{Rs}$ in these ecosystems. However, $\delta^{13}$C$_{Rs}$ was also found to vary by over 2 ‰ around this mean value over the space of hours, indicating dynamic root and microbial respiratory metabolism utilising different substrates in these soils. Furthermore, $\delta^{13}$C enrichment in ecosystem pools of $\sim$3 ‰, encompassing both bulk leaf to root metabolite and SOM gradients, resulted in average $\delta^{13}$C$_{Rs}$ being $\sim$1.5 ‰ enriched relative to the upper soil layers and $\sim$3 ‰ enriched relative to leaves and litter. These results highlight the need to understand the extent to which processes occurring during plant growth, metabolism and decomposition act to enrich plant and soil $^{13}$C following the initial photosynthetic carbon fixation. This post-fixation $^{13}$C enrichment through the ecosystem indicates the necessity to integrate SOM data over sufficient production depths in order to obtain reliable estimates of soil flux values from organic pool data. The depth-related enrichment in soil $^{13}$C, which is characteristic of many soils, was also found to contribute to $\sim$0.5 ‰ of the diel-scale variability seen in $\delta^{13}$C$_{Rs}$ through temperature-driven shifts in the contribution of different soil layers to total profile CO$_2$ production. These diurnal oscillations show that physical processes can contribute to short-term variability in the $\delta^{13}$C of soil CO$_2$ efflux, independent of any inherent shifts in the $\delta^{13}$C composition of the substrate producing CO$_2$ in the soil. Overall, the results of this study demonstrate the importance of both abiotic and biotic influences on short-term variability of $\delta^{13}$C$_{Rs}$ that need to be considered when using isotopic data for the study of carbon cycle processes and future interpretation of high resolution isotopic data sets.

Future studies on soil CO$_2$ efflux isotopic composition should address whether processes operating at longer time scales, such as variation in soil water content, changes in average soil temperature, microbial dynamics and plant phenology, affect the relative influence of abiotic and biotic processes on soil CO$_2$ efflux isotopic composition.
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